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Genetic diversity and differentiation of *Camellia euphlebia* (Theaceae) in Guangxi, China

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Camellia euphlebia is rare and endangered in China, distributed in a small region in Guangxi province. We studied the level and pattern of the genetic diversity of 84 individuals from four natural *C. euphlebia* populations by inter-simple sequence repeat (ISSR) markers. Our results indicate a relatively low level of genetic diversity in *C. euphlebia* at the species level (polymorphic loci = 55.38%, H_T = 0.1537 and H_{SP} = 0.24) and at the population level (polymorphic loci = 24.23%, H_E = 0.11), and a relatively high degree of differentiation among populations (G_{ST} = 0.3147, Shannon's index analysis = 37.5%, AMOVA analysis = 35.88%). Gene flow among populations was 0.5443. Inbreeding and limited gene flow might be the key factors resulting in the observed genetic structure of *C. euphlebia*. Strategies are proposed for the genetic conservation and management of the species.

Key words: *Camellia euphlebia*, genetic diversity, ISSR, population genetic structure

Introduction

Camellia sect. *Nitidissima* contains rare and endangered species. It is the only section whose plants have yellow flowers, and it is called "The queen of *Camellia* family" for the elegance and rareness of the plants. There are 18 species in this section (Zhang 1996), and most of them are distributed in southwestern Guangxi of China, with four in Vietnam including two endemics (Su 1994). *Camellia euphlebia* belongs to this section. It is an evergreen shrub or small tree, up to 5 m tall, and characterized by flowers having many large and golden-yellow petals with a waxy appearance.

Camellia euphlebia is distributed in Guangxi Zhuangzu Autonomous Region of China and Liangshan province of Vietnam. It mainly grows in the east of Naliang town (107°50´E) and the west of Nashuo town (108°07´E) in Fengcheng city of Guangxi. About 52 000 individuals of *C. euphlebia* are found in 68 locations covering an area of 710 hectares in these two towns (Huang 2001). A lot of wild *C. euphlebia* plants have been removed for ornamental uses.

Camellia euphlebia often grows in evergreen broad-leaved forests in valleys, by streams or on slopes at low altitudes (150–480 m) hills. The habitats have been fragmented by human

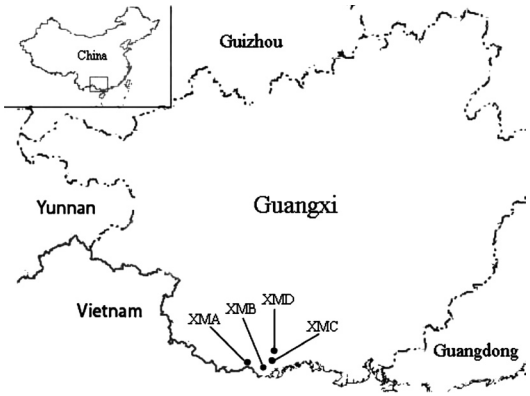

Fig. 1. Locations of the studied *Camellia euphlebia* populations.

activities, and the fruits of *C. euphlebia* are eaten by rats and other animals, resulting in a serious threat of extinction. Thus, *C. euphlebia* is classified as an endangered species in China (Fu 1995).

Molecular markers have been widely used to characterize population genetic structure of plants. These include allozymes (Culley 2001) and polymerase chain reaction (PCR) based markers like RAPD (Wachira 1995, Mirali & Nabulsi 2003), ISSR (Mondal 2002, Wang *et al.* 2004), AFLP (Paul *et al.* 1997, Zhu *et al.* 1998, Rottenberg & Parker 2003) and SSR (Rossetto *et al.* 2004). Inter-simple sequence repeats (ISSR) is a molecular marker (Zietkiewicz *et al.* 1994) that has been widely used in the studies of cultivar identification (Mondal 2002), genetic mapping (Kojima *et al.* 1998), gene tagging (Ratnaparkhe *et al.* 1998) and genetic diversity (Ge *et al.* 2003, Wang *et al.* 2004).

Studies have been conducted on the ecology (Su 1994), cytogenetics (Liao 1991), artificial breeding (Cheng *et al.* 1994) and cultivation (Zhang & Chuang 1984) of *C. euphlebia*, but not on its level of genetic diversity and pattern of genetic structure. To conserve a species, it is important to have such basic information. Therefore, we decided to examine the genetic variation within and among natural populations of *C. euphlebia* using ISSR markers. The fact that no DNA sequence information is known for the species is one of the main reasons for choosing the ISSR technique in this study. On the other hand, the ISSR markers have recently become widely used in population studies because they are highly variable, and require less investment in time, money, and labor than other methods.

Table 1. Information of the *Camellia euphlebia* populations studied.

Provenance	Pop. code	Alt. (m)	Long. (E)	Lat. (N)	Sample size
Fengcheng	XMA	160	107°53´	21°41´	21
Fengcheng	XMB	165	108°05´	21°43´	22
Fengcheng	XMC	100	108°08´	21°45´	21
Fengcheng	XMD	250	108°02´	21°38´	20

Materials and methods

Plant material

A total of 84 individuals were randomly chosen from four natural populations of *C. euphlebia* and used in this study (Table 1 and Fig. 1). Their fresh leaves were collected and dried with silica gel in zip-lock plastic bags until DNA isolation. Vouchers were collected from each population and deposited at the herbarium of Guangxi Institute of Botany (IBK), China.

DNA isolation and PCR amplification

Genomic DNA was isolated according to a modified cetyltrimethyl ammonium bromide (CTAB) procedure (Doyle 1991). One hundred primers from the University of British Columbia primer set nine were initially used for PCR amplification. Ten of them (UBC# 834: $(AG)_8$YT, 836: $(AG)_8$YA, 840: $(GA)_8$YT, 848: $(CA)_8$RG, 855: $(AC)_8$YT, 857: (AC)YG, 864: $(ATG)_6$, 866: $(CTC)_6$, 888: BDB$(CA)_7$, 891: HVH$(TG)_7$) which consistently generated clear and reproducible banding patterns were chosen for the final study.

PCR amplification was carried out in 20 μl solution containing 50 mM KCl, 2% formamide, 10 mM Tris-HCl (pH 9.0), 0.1 mM of each dNTP, 2.7 mM of MgCl2, 200 nM of primer, 20 ng of template DNA, 0.1% Triton X-100, 1.5

unit of *Taq* DNA polymerase, and double distilled water. PCR reactions were performed in a MJ Research thermalcycler as follows: initial 5 min at 94 °C, 40 cycles of 45 s at 94 °C, 45 s annealing at 51–53 °C, 1.5 min at 72 °C, and 7 min at 72 °C for a final extension. PCR amplification products and 100 bp DNA Ladder were electrophoresed on 2% agarose gels buffered with 0.5 × TBE and visualized by ethidium bromide staining. The size of the DNA fragments was identified by image analysis software for gel documentation (LabWorks Software version 3.0, UVP, Upland, CA 91786, USA).

Data analysis

Only bands that could be unambiguously scored across all the sampled populations were analyzed in this study. As a dominant marker, ISSR profiles were scored for each individual as discrete characters (presence or absence of the amplified products). Genetic diversity was measured by the percentage of polymorphic bands and Nei's genetic diversity (Nei 1973). Shannon indices of diversity, which is less influenced by sample size and its estimates of between-population diversity do not rely on Hardy-Weinberg equilibrium (Bussell 1999), were also calculated using POPGENE 1.31 (Yeh *et al*. 1999). To corroborate the *F* statistics and Shannon estimation on genetic differentiation, the nonparametric Analysis of Molecular Variance (AMOVA) program v. 1.55 (Excoffier *et al*. 1992) was used as the third approach to describe the genetic structure among populations. Treating an ISSR profile as a haplotype, AMOVA allows the estimation of population genetic parameters at the genotypic level. The significance level of this *F*-statistic analogue was determined by 1000 random permutations.

A UPGMA (unweighted pair-group method using arithmetic average) dendrogram was constructed based on the matrix of genetic distance using the SAHN clustering and TREE programs from NTSYS-pc 2.0 (Rohlf 1998). A Mantel test was performed using TFPGA program (Miller 1997) in order to test for a correlation between genetic distance and geographical distance among populations.

Results

The ISSR genetic diversity

For the four populations, the ten selected primers generated 65 bands ranging in molecular size from 270 bp to 1500 bp. On average, 6.5 polymorphic loci were scored per primer combination. The maximum and minimum number of bands observed were 9 (#840) and 4 (#857), respectively. Of these bands, 36% or 55.38% were polymorphic at the species levels. The percentages of polymorphic loci among populations ranged from 15.38% to 32.31% with an average of 24.23% (Table 2). Assuming Hardy-Weinberg equilibrium, the average gene diversity was estimated to be 0.11 within populations (H_E) and 0.1537 at the species level (H_T). Population XMA exhibited the highest level of variability (polymorphic loci = 32.31%, H_E = 0.18), while population XMC exhibited the lowest level of variability (polymorphic loci = 15.38%, H_E = 0.06). The mean Shannon's information index (H_O) of phenotypic diversity was 0.15 at the population level (H_{pop}) (Table 2), and 0.24 at the species level (H_{SP}).

Genetic differentiation

Coefficient of overall genetic differentiation (G_{ST}) among populations was 0.3147. The Shannon's diversity index analysis partitioned 37.5% of the total variation between populations (Table 3). The AMOVA analysis showed a highly significant ($p < 0.001$) genetic differentiation among the four populations of *C. euphlebia*. Of the total genetic diversity, 35.88% resided among-popula-

Table 2. Genetic variability within populations of *Camellia euphlebia*. *N* = sample size, H_E = expected heterozygosity, H_o = Shannon's information index.

Population	*N*	H_E	H_o	Polymorphic loci (%)
XMA	21	0.18 ± 0.218	0.21 ± 0.308	32.31
XMB	22	0.13 ± 0.203	0.18 ± 0.289	29.23
XMC	21	0.06 ± 0.152	0.09 ± 0.220	15.38
XMD	20	0.08 ± 0.173	0.12 ± 0.247	20.00
Mean		0.105 ± 0.187	0.15 ± 0.266	24.23

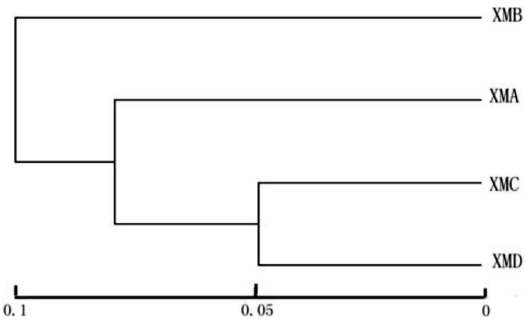

Fig. 2. UPGMA dendrogram based on Nei's genetic distance.

tions and the rest (64.12%) resided among individuals within population (Table 4).

Indirect estimates for the level of gene flow between populations were low. Gene flow (N_m) value was 0.5443 individuals per generation. Pairwise genetic identity values (I) among populations ranged from 0.9094 to 0.9586 with a mean of 0.9282 (Table 5). A dendrogram based on Nei's genetic distances showed that the XMC and XMD populations clustered together (Fig. 2). The Mantel test showed that there was no significant correlation between genetic distance and geographic distance ($r = 0.2487$, $P = 0.05$).

Discussion

Knowing the genetic variation is the first step for designing a plant conservation or genetic improvement program. This study demonstrates that ISSR-PCR offered a suitable method for the detection of genetic variability in *C. euphlebia.*

Camellia is one of the primary components of evergreen broad-leaf forest and is characterized by woody, long-lived perennials and outcrossed breeding system. High genetic diversity has been detected in some *Camellia* species, such as *C. sinensis* ($H_E = 0.320$; Li *et al.* 1996); *C. rosthorniana* ($H_E = 0.193$–0.21; Cao *et al.* 2003) and *C. japonica* ($H_E = 0.190$; Chuang & Chung 2000). Our results show that the genetic diversity in *C. euphlebia* is lower than that of those congeners at the species level and at the population level. This might have resulted from its restricted geographical distribution and isolated small populations. Geographic range is one of the major factors determining the genetic diversity of plant species (Hamrick & Godt 1989). Many studies have shown that narrowly distributed species have a lower level of genetic diversity than widespread congeners (Maki & Horie 1999, Gitzendanner & Soltis 2000). In general, a population with a small effective size tends to maintain low genetic diversity, primarily due to random genetic drift (Hartl & Clark 1997). Fragmentation and isolation of populations can result in reduced genetic variation, which may consequently reduce reproduction or survival and thereby reduce population viability (Sherwin & Moritz 2000). In our sampled areas, human activities, such as logging, removing, burning, road construction and conversion to agricultural land have caused habitat fragmentation and small, isolated populations in *C. euphlebia*.

Nei's genetic diversity, Shannon's diversity index and AMOVA detected similar genetic variation among our four *C. euphlebia* populations; it is higher than that of the congeners. For example, Kaundun *et al.* (2000) studied the structure of six tea populations by RAPD. Their AMOVA revealed that only 16% of the variance component was among populations. Wachira *et al.*

Table 3. Coefficient of genetic differentiation for *Camellia euphlebia.* — H_T = total genetic diversity, H_s = genetic diversity within population, G_{ST} = coefficient of genetic differentiation, N_m = gene flow.

Paremeter	H_T	H_s	G_{ST}	N_m
Mean	0.1537	0.1054	0.3147	0.5443
S.D.	0.0288	0.0152		

Table 4. AMOVA for four populations of *Camellia euphlebia.*

Source of variation	d.f.	Sum of squares	Mean square	Variance component	% of total variance	$P <$
Among populations	3	84.691	28.230	1.239	35.88	0.001
Within populations	80	177.190	2.215	2.215	64.12	0.001

(1995) studied the genetic diversity and relationship of 38 clones of Assam and Cambodia tea using RAPD markers and showed that 30% of the total diversity resided among populations, based on Shannon's diversity index. Paul *et al*. (1997) studied the diversity and genetic differentiation of India and Kenyan tea using AFLP. They found that 79% of the genetic variation was within and 21% among populations using Shannon's index of diversity. However, our results are in agreement with the mean G_{ST} (0.32) for dicotyledons (Nybom & Bartish 2000).

The population genetic structure of a species is also affected by a number of evolutionary factors including mating system, gene flow and seed dispersal, mode of reproduction as well as natural selection (Hamrick & Godt 1990). Limited seed and pollen dispersal contributes to low level of gene flow and high level of inter-population differentiation (Wallace 2002). *C. euphlebia* has small and discontinuous populations. Its seeds are mainly dispersed by gravity. It is mainly pollinated by insects whose migratory distances are small, and some self-pollination is known to happen (Cheng *et al*. 1994). All these would promote inbreeding within and reduce gene flow among *C. euphlebia* populations. Therefore, inbreeding and limited gene flow are at least two key factors contributing to the high differentiation observed among the *C. euphlebia* populations in this study.

The aim of conservation is to preserve existing genetic diversity and the potential of a species to evolve (Hopper & Coates 1990). Any conservation program should ensure the long-term survival of a species and maintain its ecological and evolutionary processes, and both of these require the preservation of genetic variation (Hamrick & Godt 1996). Because *C. euphlebia* has isolated small populations and high genetic variation among populations, we believe all the existing populations should be protected *in situ* free from anthropogenic and agricultural impacts in order to preserve as much genetic variation as possible. We should find ways to enhance gene flow among *C. euphlebia* populations, such as cross-transplanting individuals among populations. Furthermore, we should collect samples from all the natural populations and develop methods for *ex situ* conservation of the genetic resources for *C. euphlebia*.

Table 5. Nei's (1972) original measures of genetic identity (above diagonal) and genetic distance (below diagonal).

	XMA	XMB	XMC	XMD
XMA		0.9212	0.9325	0.9327
XMB	0.0821		0.9146	0.9094
XMC	0.0699	0.0893		0.9586
XMD	0.0697	0.0950	0.0423	

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